

EXPERIMENTAL APPROACH
TO THE PATHOGENESIS
OF RETROLENTAL FIBROPLASIA

BY

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The Discussion is Based on the Following Papers:

- I. GYLLENSTEN, L. J. and HELLSTRÖM, B. E.: Experimental approach to the pathogenesis of retrolental fibroplasia. I. Changes of the eye induced by exposure of newborn mice to concentrated oxygen. *Acta pædiat.*, 43, Suppl. 100: 131, 1954.
- II. GYLLENSTEN, L. J. and HELLSTRÖM, B. E.: Experimental approach to the pathogenesis of retrolental fibroplasia. II. The influence of the developmental maturity on oxygen-induced changes in the mouse eye. *Am. J. Ophth.*, 39: 475, 1955.
- III. GYLLENSTEN, L. J. and HELLSTRÖM, B. E.: Experimental approach to the pathogenesis of retrolental fibroplasia. III. Changes of the eye induced by exposure of newborn mice to general hypoxia. *Brit. J. Ophth.*, 39: 409, 1955.
- IV. GYLLENSTEN, L. J. and HELLSTRÖM, B. E.: Experimental approach to the pathogenesis of retrolental fibroplasia. IV. The effects of gradual and of rapid transfer from concentrated oxygen to normal air on the oxygen-induced changes in the eyes of young mice. *Am. J. Ophth.*, April, 1956.
- V. HELLSTRÖM, B. E.: Experimental approach to the pathogenesis of retrolental fibroplasia. V. The influence of the state of nutrition on oxygen-induced changes in the mouse eye. *Acta pædiat.*, 45:43, 1956.
- VI. HELLSTRÖM, B. E.: Experimental approach to the pathogenesis of retrolental fibroplasia. VI. The influence of the oxygen concentration on the oxygen-induced changes in the mouse eye. *Acta pædiat.*, in press.
- VII. HELLSTRÖM, B. E.: Experimental approach to the pathogenesis of retrolental fibroplasia. VII. Effect of oxygen exposure on the electroretinogram in kittens. *Arch. Ophth.*, February, 1956.
- VIII. HELLSTRÖM, B. E.: Experimental approach to the pathogenesis of retrolental fibroplasia. VIII. The production of irreversible, severe eye changes through intense and prolonged oxygen exposure in mice. *Acta pædiat.*, in press.
- IX. HELLSTRÖM, B. E.: Experimental approach to the pathogenesis of retrolental fibroplasia. IX. The histochemical localization of succinic dehydrogenase in the retina of normal and oxygen-exposed animals. *Acta Pathol. et Microbiol. Scand.*, in press.
- X. HELLSTRÖM, B. E.: Experimental approach to the pathogenesis of retrolental fibroplasia. X. In vitro metabolism of the retina of oxygen-exposed ratlings. Stockholm 1956.

References to these papers are indicated by the Roman figures given above.



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A. Introduction

Since retrolental fibroplasia (RLF) was described as a clinical entity for the first time by Terry (88) in 1942, the disease has been recognized in different parts of the world, in some places constituting a common cause of blindness. It is natural that the disastrous final result of the disease in its more severe forms has mobilized the interest of ophthalmologists, pathologists and pediatricians with an intense search for its cause and the possibilities of therapy and prevention. Aetiological speculations have covered almost all conceivable pre- and postnatal factors (reviews by *e.g.* Bjelkhagen, 9; Zacharias, 96; Castrén, 19). Most of the early work in this field, however, either gave negative results or, if reported as positive, could not be confirmed in later investigations. This was the experience also regarding attempts to reproduce the disease in animals.

The first animal experiments dealing with the effect of exposure to high concentrations of oxygen on the immature eye were published in a preliminary form in 1952 by Gyllensten and Hellström (35). They were initiated by clinical observations on the correlation between the treatment of premature children with high oxygen in the incubators and the incidence of the disease (Kinsey and Zacharias, 58; Campbell, 18; Crosse, 22) and showed that intermittently administered concentrated oxygen caused changes of the eyes of full-term young mice, in some details resembling the pathological picture of RLF.

During subsequent years more and more convincing facts accumulated from both clinical studies and animal experimentation, which incriminated oxygen as the main environmental factor connected with the production of the disease. Today it is almost generally agreed that this connection is definitely established, and as a practical consequence restrictions of the oxygen treatment of premature infants have been adopted, leading to a reduced incidence of the disease (*e.g.* Campbell, 18; Ryan, 83; Lanman, 61). In spite of these encouraging results, there is as yet no complete agreement concerning the mechanism of oxygen effect.

The purpose of the present investigation was to confirm the work preliminarily reported and, by extending these morphological studies and by exploring the oxygen effect also with physiological methods, it was hoped that the information gained would be useful towards a more basic under-

standing of the oxygen effect on the animal retina. Such information would most likely be clinically applicable as to the pathogenesis of retrolental fibroplasia.

B. Review of Animal Experimentation as to Possible Aetiological Factors Other than High Oxygen Exposure

Before the experiments on high oxygen effect are dealt with, a short account will be given concerning animal experimentation referring to other aetiological factors including our experiments as to the influence on the retina by general hypoxia.

1. *Other Investigators*

Terry (89) who interpreted RLF as an abnormal persistence of hyaloid vessels and of the tunica vasculosa lentis, also made attempts to produce the disease in animals. In these experiments, incompletely reported, he also tried to cause a precocious involution of the hyaloid artery and tunica vasculosa system in opossums and young rats. The disease was not produced, but it appeared that repeated dilatation of the pupil and the ophthalmoscopical examination tended to delay the time at which this vascular system disappeared.

Vitamin E was in the focus of interest for some time since Owens and Owens (71) postulated that the disease had some connection with vitamin E deficiency. Observations by Callison and Orent-Keiles (16) indicated that ocular abnormalities could be induced in young rats by vitamin E deficient mothers, and that they consisted of smaller eye-balls than normal, eyelids which failed to open, an opaque white membrane behind the pupil and in one instance a large clot of blood completely filling the pupil. No histological examination was made. The experiments were later repeated by Patz (76), but the results could not be confirmed. Further clinical trials have also failed to establish the significance of vitamin E deficiency (*e.g.* Kinsey, 56; Bosquet and Laupus, 12; LaMotte and Tyner, 60; Reese, 80).

Warkany and Schraffenberger (93) reported eye defects in the offspring of vitamin A deficient rats, a finding confirmed by Jackson and Kinsey (50). However, the administration of large doses of vitamin A failed to prevent the development of RLF (Clifford and Weller, 20).

Hepner (41) was able to maintain the embryonic vascular supply of the lens in kittens by filling the extracellular fluid spaces by means of massive infusions and blood transfusions. Furthermore, capillary angioma-like structures with mild surrounding inflammatory cell infiltrations in the periphery of the retina were described. They were not seen to enter the

vitreous body, but the whole retina was sometimes seen to bulge towards this body. Based on clinical studies Hepner and Krause (42) claimed that a similar mechanism may be responsible for the production of RLF in premature infants. This opinion was supported by Pallotta (74) but other authors stated that blood transfusions were not related to the occurrence of RLF (*e.g.* Unsworth, 92; Ryan, 83; Dancis *et al.*, 24). Hepner *et al.* (43) were not able to confirm the deleterious effect of high electrolyte content of feedings. They found that neither the incidence nor the severity of acute RLF were decreased significantly by feeding the infants with either human milk or cows milk mixtures with electrolytes adjusted to human milk levels.

The conception that pre- or postnatal general hypoxia is a factor of importance has been held by several investigators. Ingalls *et al.* (49) exposed pregnant mice to subnormal atmospheric pressures at different stages of gestation. A small number of the offspring showed the open-eye syndrome which, apart from the defective formation of the eyelids, included distortions of the developing retina with folding, retrogressive changes within the lens, as well as proliferation of vasoformative tissue both in the inner vascular layer of the retina and in the retrolental space. Two of the control animals, however, also developed the syndrome. Similar abnormalities had been produced in the offspring of rats through low pressure hypoxia by Werthemann and Reiniger (94), but they did not associate their findings with the pathology or aetiology of RLF. Nor did Campbell (17) who is often quoted in this connection. Using the method of India ink injection in rats he demonstrated that postnatal low pressure hypoxia caused a decrease in the calibre of retinal vessels, furthermore, that the capillary bed was less dense than normally and that the so-called capillary-free zone around the arteries was found to be less well defined and narrower than in control animals. It was postulated that the oxygen tension in the tissues surrounding the developing vascular bed controlled the mode of formation of the developing vessels. The pattern of the extended abnormal vascularization was not described as being similar to that found in the vascular pathology of RLF (Ashton, 1). Moreover the existence of a real capillary-free zone around the arteries as it is demonstrated with the India ink injection has been doubted (Janes and Bounds Jr., 51). Ashton *et al.* (4) described engorgement of retinal vessels in kittens as a result of continuous hypoxia at the 10–15 per cent oxygen level for periods of 4 to 13 days. The peri-arterial capillary-free zones were narrowed. Some increase of angioblastic activity was found but no intravitreal proliferation, and it was concluded that the increase in angioblastic activity was probably within the upper limits of normality, Patz (75, 76) raised mice and rats from one to

four days of age continuously with 10–14 per cent oxygen concentrations and also found a narrowing of the capillary-free zone and a denser capillary network in the retinal periphery.

2. *Present Experiments as to the Effect of General Hypoxia (III)*

It was thought necessary to attempt to increase the knowledge concerning the influence of postnatal general hypoxia on the immature retina, as the previous investigations mentioned comprised relatively small materials and the degree of hypoxic insult was not severe.

Newborn mice were exposed to severe, intermittent general hypoxia with oxygen concentrations as low as close to 3 per cent. This must be considered a maximal hypoxic injury and it was seriously impairing the general condition of the animals with a high mortality. The treatment was continued with some animals up to an age of three weeks. The only pathologic findings of the retinae were haemorrhages in the nerve-fibre layer, occasionally breaking through the internal limiting membrane into the vitreous body. No hyperplastic changes of the retinal vessels, as judged by the formation of capillary whorls or more diffuse proliferations, were observed.

3. *Clinical Experience as to the Rôle of General Hypoxia and Applicability of Animal Experiments*

No convincing clinical evidence has so far been presented indicating that general hypoxia *without preceding oxygen therapy* may be responsible for advanced stages of RLF, although this mechanism has been suggested (Ingalls, 47; Szewczyk, 86). On the other hand, clinical observations have been made by several investigators that the "relative hypoxia", postulated to follow rapid withdrawal from oxygen exposure, is in some way connected with the first appearance or progression of the disease (Jefferson, 52; Szewczyk, 87; Bedrossian *et al.*, 6, 7). A similar mechanism has been suggested if the oxygenation is impaired for some reason (Ingalls, 47; Manschot, 65). As attempts have been made in the present study to approach these problems by animal experimentation, they will be dealt with separately later on.

Retinal and vitreous haemorrhages are common findings in RLF but, on the other hand, they occur also in the retinae of premature children without any other sign of the disease (Tyner, 90). This occurrence following experimental hypoxia does not justify conclusions as to the significance of general hypoxia in the aetiology of RLF.

It is possible that the effect of more continuous and moderately intense hypoxia might include changes more similar to the early stages of RLF,

but so far, the experimental work has failed to give any positive evidence that *general* hypoxia should be the only factor of importance in the aetiology of RLF.

In the experimental work reviewed above the attempts to produce lesions of the animal eye definitely similar to RLF have failed and no positive information concerning its aetiology has been gained.

C. Animal Experimentation as to the Early Effect of Oxygen Exposure on the Eye

1. *Present Investigation (I).*

The preliminary observations (Gyllensten and Hellström, 35) were extended to an investigation, where the effect of oxygen on the immature eye at a concentration of close to 100 per cent was studied. In this, as in most of the subsequent morphological work, newborn mice were used. At birth the retina of this animal is highly immature, the hyaloid vessels persist, the nuclear layers are not differentiated, the bacillary layer is absent and the whole process of vascularization of the retina through the outgrowth of vessels from the optic disc takes place postnatally. The maturation of the eye takes a relatively short time; the outgrowth of retinal vessels is completed within 10–11 days, as is the differentiation in outer and inner nuclear layers. During the same time hyaloid vessels regress and the bacillary layer is formed, with the outer and inner segments being successively evident. The retinal vessels are initially found only as a superficial net in the nerve-fibre layer, but later on the deep net is formed on both sides of the inner nuclear layer.

The process of retinal vascularization has been studied in more detail in several species by Michaelson (66, 68), the conditions in mice and rats being greatly similar. For detailed studies with quantitative measurements the injection technique used by him is suitable. The routine histologic method used in the present investigation, however, seems to allow of sufficient information as to the degree of vascularization and the occurrence of vascular pathology.

The effect of continuously and intermittently administered oxygen of variable duration was studied. It was found that continuous oxygen exposure caused an almost complete inhibition of the outgrowth of retinal vessels. Occasional spindle-shaped cells were seen in the nerve-fibre layer but no capillary lumina and no vascular proliferations. In contrast the hyaloid vessels in the vitreous body showed an abnormal persistence and even a proliferation. The continuous exposure was not maintained for a longer time than maximum 16 days and other changes of retinal structures were not conspicuous.

In another group of animals the interest was focussed on the phase following transfer to air after 5 days oxygen exposure. These findings were of special interest with regard to their close resemblance to the early pathology of RLF. A few days after the transfer the delayed vascularization of the retina had started at the papilla and the vessels spread successively towards the periphery. Almost regularly, however, the vessels appearing showed an abnormal pattern. They were decidedly hyperplastic, especially in the nerve-fibre layer, making this layer to appear thickened, especially in the region of the optic disc. The abnormal vessels had markedly varying calibres and frequently formed angioma- or glomerular-like whorls, which were seen budding towards the vitreous body, sometimes breaking through the internal limiting membrane. The retinal vessels belonging to the deeper layer did not appear to proliferate in the same manner.

Small haemorrhages located in the nerve-fibre layer were common and vitreous bleedings sometimes originated from dilated retinal vessels. The more common source of these vitreous haemorrhages were, however, the hyaloid vessels.

Together with this vascular pathology changes of the other retinal structures were found, such as thickening of the central part with irregularities and occasional folds.

The dominating feature of the oxygen-induced early changes in the eyes of immature mice is the inhibition of retinal vascularization during oxygen exposure, followed by abnormal proliferations on transfer to air.

2. Other Investigators

When these results are compared with those of other investigators there is generally good concordance. Patz (75, 76), however, observed local proliferations of the retinal vessels during continuous exposure. This discordance can, however, be explained by the lower concentrations of oxygen used by him, as will be discussed later. The agreement with the experimental work by Ashton *et al.* (4) is striking. Here the effect of oxygen on developing retinal vessels was studied in kittens, mainly by means of injection technique. The stage of vascularization at birth is more advanced in this animal than in mice, but the outgrowth of retinal vessels normally continues during the first 3 weeks, postnatally. It was reported that high concentrations of oxygen were able to obliterate the ingrowing vessels, the effect being either complete or partial. The process commenced as marked constriction of the arteries and arterioles, beginning peripherally, where the vessels were most immature. This vaso-obliterative effect of hyperoxia was also demonstrated in the developing retinal vessels of the young rabbit and ratling, although to a much less marked extent.

On transfer to air a revascularization began and this phase of vasoproliferation was abnormal in character, forming a richer plexus than normal with outgrowth of numerous corkscrew proliferations extending into the vitreous body as fine arborizations or as glomerular tufts. No apparent degenerative change in the retinal neural elements were reported.

Using a limbal window technique Ashton and Cook (2) directly observed the constrictive and obliterative effect of oxygen, also in kittens. An immediate effect was apparent after only five minutes of exposure to hyperoxia and the delayed effect completely obliterated the whole retinal circulation after an exposure of only eight hours' duration.

Michaelson *et al.* (68) concluded from India ink-injected retinae of oxygen-exposed young mice that the most prominent change was a delay in the formation of the capillary bed situated around the disc. The extent of the vascular outgrowth from the disc was not grossly affected. These findings are in definite discordance with the results of present studies, probably due to the lower concentrations of oxygen used and the relatively small material of the former investigation.

Gerschman *et al.* (32) in experiments with oxygen-exposed young mice noted the engorgement and proliferation of the tunica vasculosa lentis with vitreous haemorrhages and also pointed out the reciprocal relation between the size of the retinal network and that of the tunica vasculosa lentis.

3. *Histopathology of Early RLF*

Before the experimental findings now mentioned are evaluated a short review of the early pathology of RLF is necessary. This condition has been described by several investigators (*e.g.* Friedenwald *et al.*, 31; Heath, 37; Tyner and Frayer, 91; Reese *et al.*, 82; Ashton, 1) and changes of the retinal vessels form the centre of interest. Proliferations are noted in the equatorial region of the nerve-fibre layer of the retina and described as small nests of endothelial cells and increase of glial elements (Reese *et al.*, 82). Subsequently the endothelial cells canalize with surrounding haemorrhages and oedema and masses of newly formed capillaries are seen sprouting into the vitreous body, breaking through the internal limiting membrane or sometimes growing along the surface of the retina. The other structures of the retina are not conspicuously abnormal in these early stages of the disease. Definite signs of inflammatory cell reaction are absent.

Ashton (1) observed the same type of glomerular tufts in pathologic specimens from early RLF as in the experimentally induced lesions. As stated previously by Reese *et al.* (82) the oral region was not primarily involved and the process was as a rule confined to the nerve-fibre layer of the retina in the equatorial region. Only in later stages did the exuberant

vasoformative tissue extend into the ora. The primary location coincides with the region the outgrowing vessels have reached in the premature infant. The spindle-shaped cells, occurring together with endothelial cells and vessels, earlier interpreted as glial cells (Friedenwald *et al.*, 31; Reese *et al.*, 82), were shown to occur together with PAS-positive granules. These cells are present, but less abundantly in the normal retina, where they enter before the vascular ingrowth. The PAS-positive granules occurring together with these spindle cells have the same histochemical properties as glycogen (Serpell, 85). On the basis of these findings Ashton (1) concludes that the cells are mesenchymal precursors of the retinal vascular system and are claimed to originate from the mesodermal coats of the hyaloid artery.

4. Comparison between the Oxygen-Induced Early Changes of the Animal Eye and Early Stages of RLF

There seems to be convincing evidence that the early pathology of RLF shows detailed concordance with the changes found in the vasoproliferative phase of the oxygen-induced lesions of the animal eye. Both conditions have as their main feature the abnormal, overgrowing angioblastic activity of vessels and vascular precursors in the nerve-fibre layer occurring either as a more diffuse infiltration or as localized glomerular tufts budding towards the vitreous body. PAS-positive granules occur abundantly both in RLF and in the experimental disease (Serpell, 85; Patz, 75, 76).

As for the vasoconstrictive and vaso-obliterative phase of the experimental disease this has not been recognized in descriptions of the early histopathology in RLF. No special attempts have been made, however, to disclose with histopathologic method as to whether vaso-obliteration is present in the eyes of an oxygen-treated premature infant. Such an investigation, however, must encounter great difficulties, inclusive of the lack of suitable pathological specimens, the special technique necessary etc. Clinical material has shown, on the other hand, that oxygen inhalation causes constriction of the retinal vessels of premature infants (Bedrossian, 6, 7; Huggert, 44, 45) and that a narrowing of retinal vessels frequently precedes the vascular proliferations in RLF (LaMotte, 59; LaMotte and Tyner, 60; Huggert, 44, 45; Patz, 75). In Huggert's material it was possible to show the extremely narrow vessels only in cases that had been given an extra supply of oxygen. A transition from these narrow vessels and white papillae to wide vessels and grey papillae took place, as a rule, within a few days after discontinuing the administration of oxygen. The connection between the cessation of oxygen treatment and the appearance of retinal oedema was not quite so unmistakable.

It does not seem improbable that long-standing oxygen exposure may lead to obliterative changes of the retinal vessels in premature infants also.

In spite of the striking and convincing similarities between the early stages of RLF it is unjustifiable to state emphatically that they are identical and to conclude that RLF is caused exclusively by oxygen exposure. One principal objection concerns the questionable applicability of experiences from animal experiments on diseases in man. Furthermore, although the lesions produced by oxygen exposure in the animal eye seem typical, this does not mean that they are specific only for oxygen damage. The morphological changes may just represent the way the immature retina and its vessels react on the various physico-chemical injuries impairing circulation and nutrition. This is more than a theoretic possibility as shown in recent studies by Ashton and Cook (3a), where the same type of glomerular-like capillary proliferation, as seen in the oxygen-induced disease, was produced through artificially induced detachments without oxygen exposure.

Another objection to the acceptance of a complete identity between RLF and the experimental lesions concerns the different outcome of the respective diseases.

D. Late Stages of the Oxygen-Induced Disease in the Animal Eye

1. *Present Investigation (VIII).*

As most investigations dealing with the oxygen-induced disease of the animal eye showed that the lesions were reversible in contrast to the cicatricial stages found in human RLF, it was thought to be of interest to intensify the oxygen exposure through prolonged, intermittent administration.

Slight atrophic changes had been noted to still persist 6 months following only 5 days exposure to concentrated oxygen in the present investigations. In this extended study, where the exposure was continued intermittently for 1-3 months, the atrophic changes of the retina were sometimes found to be severe. The inner vitreal layers were most frequently affected, but in some animals the atrophy included also the outer layers, reducing the retina to only a few rows of intermixed cells. The vascular proliferations, characteristic of the early stages, disappeared in 2 months and slight attempts to organize the vitreous haemorrhages subsided. Retinal detachment with proliferating pigment epithelium occurred but were only shallow and not so frequent. A complete detachment of the retina did not occur.

It was thus possible to produce severe, irreversible, atrophic changes of the immature mouse eye through intense, concentrated oxygen exposure.

2. Other Investigators

Patz (75, 76) reported detachment occurring in two oxygen-exposed puppies of the twenty studied but no residual lesions in the other animals. Nor has Ashton *et al.* (4) succeeded in producing a cicatricial stage of the disease in kittens. Gerschman *et al.* (32) described atrophy of the inner layers in oxygen-exposed newborn mice, similar to those mentioned above.

3. Histopathology of Cicatricial RLF

The cicatricial stage has been described from a pathological point of view *e.g.* by Reese *et al.* (82). The vitreous body is invaded by angiomatous tissue, the haemorrhages occurring from the newly formed vessels are organized and the fibrovascular tissue in the vitreous body tends to shrink, which contributes to the folding and detachment of the retina and in the final stages of the severe cases, the greater part of the retina is folded and detached by this contracture. Secondly, complications such as synechias, glaucoma and atrophy of the whole bulb usually follows. Retinal detachment may also, at least in part, result from a transudative process (Friedenwald, 30). These late cicatricial stages are not specific for RLF but the fibrotic remains of the glomerular tufts are sometimes recognizable.

4. Comparison between the Oxygen-Induced Late Changes of the Animal Eye and cicatricial Stages of RLF

It is thus obvious that the final stages of RLF and the disease in mice differ widely. In the former the fibro-vascular organization of the vitreous body with retinal detachment is the outstanding feature, in the latter atrophic changes dominate and occur mainly following such intense exposure. This does not definitely contradict that both diseases may have the same cause, although differences between the human and animal eye as to structure, maturation and mode of reaction account for the discordance. The information gained from this part of the investigation seems in any case of value. Prolonged and intense oxygen exposure is able to cause severe atrophic changes of the mouse eye, seriously impairing its function.

E. Clinical Experience as to the Rôle of Oxygen Exposure

There is no longer need to base on animal experiments the opinion that oxygen exposure is intimately associated with the occurrence of RLF, for direct overwhelming evidence has accumulated during recent years indicating such a relationship (Campbell, 18; Crosse, 22; Crosse and Evans, 23; Ryan, 83; Goldman and Tobler, 33; Jefferson, 52; Bedrossian *et al.*, 6, 7; Forrester *et al.*, 29; Locke, 63; Gordon *et al.*, 34; Brændstrup and Flensburg,

13; Blix, 11, Huggert, 46). Of special importance have been the controlled studies reported by Patz *et al.*, (77) Lanman *et al.* (62) and Kinsey *et al.* (57). The results from the last cooperative study have so far been reported only preliminarily, but the data indicated that the incidence of RLF was positively associated with the use of oxygen, and much of the deleterious effect appeared to be associated with exposure to oxygen within the first ten days of life. Limiting the amount of oxygen used in the care of premature infants to that required for clinical emergency did not influence the survival rate.

With this knowledge in mind, further animal experimentation may still be of value to help to find out *how* oxygen exerts its injurious effects, rather than *if* it does so. In the following are summarized some experiments that have been made to investigate factors that may modify the oxygen injury, and attempts were also made to explore the mechanism of the oxygen effect.

F. Factors Influencing the Oxygen-Induced Disease in Animals

F1. The Influence of the Maturity of the Retina

1. *Present Investigation (II).*

In this part of the study the effect of concentrated oxygen of short duration on the early vasoproliferative changes in young mice of different ages was investigated. Three age groups were selected: newborn and animals 5 and 10 days of age, the last group representing a developmental stage, where the normal vascularization of the retina is nearly completed. The proliferating changes of the retinal vessels with and without budding into the vitreous body were more frequently seen in the two first age groups, as compared with the oldest age group. It took a longer time for them to develop in the newborn group, and in the two other groups they were not, as in the newborn, concentrated around the disc, but had shifted to the periphery of the retina. Consequently this type of vascular reaction occurred, where the vessels were at a sensitive developmental stage of growth. A remarkable finding was the very high incidence of atrophy of the inner layers of the retina in the oldest age group together with the less intense vascular proliferation, whereas atrophy did not occur among the newborn, where the vascular reaction was more intense. This difference seems to indicate an inverse relationship between the occurrence of proliferating vessels and atrophy and is compatible with the hypothesis that these proliferations represent a reactive, possibly reparative mechanism. There is also the possibility that these high oxygen concentrations directly injure retinal cell elements and in such case the older animals would be more sensitive. This possibility

is to some degree supported by the findings of Noell (69), who succeeded in producing a selective visual cell damage by exposing adult rabbits to high concentrations of oxygen, no vascular changes being reported. In the present study with mice, however, the inner layers were usually affected, leaving the visual cells intact.

2. Other Investigators

The influence of age concerning the degree of vaso-obliteration following oxygen exposure was studied by Ashton *et al.* (4) in experiments with kittens. The severity of vaso-obliteration was inversely proportional to the maturity of the vessels, and obliteration could not be produced once the retinal vascularization was mature. Patz (75, 76) also points out that the animal's eye is susceptible to oxygen only when the retina is incompletely vascularized and is resistant once vascularization is complete. Gerschman *et al.* (32) used animals of different ages but no conclusion as to the influence of maturity may be drawn due to the variable durations of exposure among the different groups.

From the information thus collected it seems clear that in different species the sensitivity of the retinal vessels to the oxygen effect is confined to the developing stage of these vessels. This is true both of vaso-oblitative and vasoproliferative phases.

3. Clinical Experience and Applicability of Animal Experiments

The relationship between oxygen sensitivity and retinal vascular immaturity thus demonstrated is in agreement with the incidence of RLF in premature infants being inversely related to gestational age (*e.g.* Unsworth, 92; King, 55).

F². The Influence of the Oxygen Concentration

1. Present Investigation (VI).

In the present investigation, so far dealt with, the oxygen concentration has been close to 100 per cent. This is a concentration, at which adult mice survive only a few days, whereas newborn mice may for some reason tolerate it for several weeks. The intolerance of the adult animal is due to the general toxic effect of oxygen, where pulmonary changes with respiratory insufficiency is part of the picture. The better tolerance of the newborn seems paradoxical in view of the more dominating rôle the anaerobic metabolism plays in this age group (Fazekas *et al.*, 28). At lower concentrations, *e.g.* below 70–80 per cent, a prolonged stay is possible also for adult animals, without impairment of the general condition.

It seemed of interest to investigate the oxygen effect with concentrations,

where probably the eyes were more selectively affected and to explore the correlation between frequency and intensity on the one hand, and the oxygen concentration on the other. Concentrations at the levels of 30, 40, 50, 60, 70 and 80 per cent were used. There were mainly two groups arranged with different durations of exposure, one with 5 days of oxygen followed by 10 day's stay in air, the other with 30 days of continuous exposure. The former group allowed special consideration to be given to the vasoproliferative stage of the disease, the latter afforded information as to the incidence of atrophic changes with prolonged exposure.

In the short-exposure group the same type of eye pathology was encountered, as seen when concentrated oxygen was used. This occurred at a very high incidence with concentrations from 50 per cent and above. Below 50 per cent the frequency rapidly decreased, but still at 40 per cent these eye changes were not rare. At 30 per cent all eyes were normal. There was no constant trend indicating an increased severity with increasing concentration above 40 per cent. As for the prolonged continuous group, the same tendency was found. At 30 per cent the histologic picture of the eyes was entirely normal, at 40 per cent the risk was still fairly low, but at higher concentrations it rapidly increased. An inhibited retinal vascularization was noted more regularly and completely at higher concentrations, and there was a close relationship between the incidence of retinal vascular inhibition and atrophic changes. On the other hand, there was no obviously increased intensity of the atrophic changes with higher concentrations. Other important information was gained at the continuous exposure with lower concentrations. In animals, where the inhibition of retinal vascularization was not complete, localized vascular proliferations were seen in the nerve-fibre layer. Consequently, at these lower concentrations, the vasoproliferative stage does not always coincide with a decrease of the oxygen concentrations as on transfer to air. This is in accordance with the experiences reported by Patz *et al.* (78). These vascular proliferations seen during continuous exposure were, however, much less frequent and less intense than when the oxygen environment was lowered.

From this investigation it seems clear that the injurious oxygen effect on the retina of the newborn mouse starts somewhere between 30 and 40 per cent and that the margin of safety seems to be narrow.

2. Other Investigators

The work by Ashton *et al.* (4) included four different levels of oxygen concentration as regards the vaso-obliterative phase. The results were in good agreement with those of the present investigation. Severe obliteration

was found with 35-55 per cent and doubtful or absent with 25-35 per cent. Patz (75, 76) has stated that oxygen concentrations below 40 per cent had no effect on the retinal vessels.

3. *Clinical Experience and Applicability of Animal Experiments*

In some of the controlled clinical RLF-studies the "curtailed" group received oxygen at a concentration of 40 per cent. The results of the animal experimentation indicates that this is a concentration which is not safe, at least not for the immature animal eye.

F³. The Influence of the Duration of Oxygen Exposure

1. *Present Investigation*

In a great part of the present investigation the duration of oxygen exposure was 5 days and this period was sufficient to cause conspicuous eye pathology. No systematic study was made to investigate the minimal time necessary to cause pathologic changes.

2. *Other Investigators*

Ashton *et al.* (4) state that in the kitten the severity of vaso-obliteration is directly related to the duration of exposure and that it takes about 36 hours to obtain total obliteration as judged by injection preparations. Further experience seemed to indicate that only a few hours of exposure were requisite to produce an obliteration not completely reversible, as judged by direct observation with the use of the limbal window technique (Ashton and Cook, 2). Patz *et al.* (78) showed that the incidence of ocular lesions in oxygen-treated mice increased multifariously, when the exposure was extended from 1 to 2 weeks and even more, with treatment for 3 weeks.

3. *Clinical Experience and Applicability of Animal Experiments*

From the results reported by the cooperative study it was concluded that the risk of developing RLF appeared to increase with exposure to oxygen during the first 10 days of life.

Thus it seems justifiable to conclude both from clinical and experimental work that there is a positive relationship between the duration of oxygen treatment and the incidence and severity of eye changes, but that only a few days of exposure is probably sufficient to cause permanent eye damage.

F⁴. The Influence of the Mode of Transfer to Air Following Oxygen Exposure

There has been much discussion concerning the rôle played by the mode of transfer to air following the oxygen exposure. It has been postulated

(Szewczyk, 86, 87) that oxygen exposure merely causes an adaptation of the retina to higher oxygen tensions without any structural changes and, when oxygen suddenly is withdrawn, a state of "relative hypoxia" of the retina follows with ensuing proliferations of retinal vessels. Another group of investigators (Patz *et al.*, 77; Gordon *et al.*, 34; Lanman *et al.*, 62) represent the opinion that oxygen is injurious during continuous exposure either owing to ischaemia or histotoxic anoxia. A lowering of the oxygen environment may possibly exaggerate this hypoxic injury. The vasoproliferative phases occurring in animal experiments (Ashton *et al.*, 4; I) on transfer to air clearly indicated that a connection existed between the lowering of oxygen and overgrowth of retinal vessels. It was thought of value to find out whether this reaction and the other eye changes could be modified by a process of successive lowering of the oxygen concentrations.

1. *Present Investigation (IV).*

In this part of the study also performed with mice, the eye changes were compared in different groups subjected either to rapid or to gradual diminishing of oxygen concentration. It was found that the oxygen-induced disease of the mouse eye could not be prevented by the gradual transfer to air, as performed in these experiments. Some differences were encountered, however, but mostly regarding the time schedule of the changes. The appearance of pathological vascularization of the nerve-fibre layer was initially delayed, but the more prolonged observation disclosed that this difference was levelled. The changes of hyaloid vessels, the irregularities and atrophies of retinal layers were not significantly influenced by the method of transfer to air.

With the present knowledge that concentrations of 60 to 80 per cent are almost as effective as concentrated oxygen in producing inhibition and obliteration of retinal vessels it would probably have been more rational to decrease the concentration to the 40-50 per cent level as the first step in the process of weaning. Doing so it might have been possible to modify the eye pathology more, but it is highly improbable that such a procedure would have prevented the eye changes entirely.

2. *Other Investigators*

Patz *et al.* (78) observed vasoproliferative changes occurring in the immature animal eye during continuous oxygen exposure and concluded that a lowering of the environmental oxygen was not essential for this phase to appear. It was, however, agreed that it was reasonable to assume that rapid withdrawal might further accentuate the injurious effect. In some of the experiments by Ashton *et al.* (4) the animals were transferred from high oxygen

concentrations to an environment with lower concentrations than air. This did not intensify the vasoproliferation.

To summarize the animal experiments dealing with this problem it may be concluded that the vasoproliferative phase regularly occurs on transfer to air and exceptionally during continuous oxygen exposure. It can only partly be influenced by the mode of transfer to air, as, if this is made gradually, its appearance is delayed. Thus the course of events leading to ocular pathology seems to be more determined by the stay in oxygen than by the method of transfer to air.

3. *Clinical Experience and Applicability of Animal Experiments*

There is a probability that a process of gradual weaning is more apt to modify the disease in the premature infants than in animals. As already mentioned, Szewczyk (86, 87) has advocated that RLF is a pure "withdrawal disease" and has based his opinion not only on its appearance in connection with transfer to air, but also on the presumptively favourable results of oxygen therapy with regression of early changes of RLF. This view is also supported by Manschot (65) and gains additional support from the studies made by Bedrossian *et al.* (7), who reported a significantly higher incidence of the disease in infants suddenly removed from an atmosphere of 50 per cent oxygen as compared with a group where oxygen was gradually reduced from the same initial concentration. The cooperative study group (57), on the other hand, was reluctant to accept the view that a slow weaning process is of benefit and pointed out that, since the incidence of RLF appears to increase with each additional day of exposure, it follows that prolonging the stay of the infant in oxygen to permit slow weaning may increase the risk of developing the disease.

Additional clinical data from controlled studies seem necessary before this question may be definitely settled. It should be pointed out that there is a margin between the oxygen content of air and the 30–35 per cent level, below which oxygen has not been proved to exert any injurious effect, a margin suitable to use in slow weaning tests.

F5. The Influence of the State of Nutrition

1. *Present Investigation (V).*

This part of the investigation was undertaken for the following reasons. Some of the animals exposed to oxygen showed a relatively slow gain of weight indicating the need of exploring the influence through the state of nutrition *per se*. The experiments were extended to include both the influence on normal vascularization and differentiation, and on the oxygen-induced pathological vascularization as well.

It was shown that lessened nutrition leads to a certain delay of the differentiation of retinal layers and of the vascularization of the retina. This retardation of the outgrowth of vessels, however, was only slight as compared with the inhibition following oxygen exposure. In the oxygen-exposed animals the state of nutrition did not obviously influence this oxygen-induced inhibition. The vasoproliferative phase, on the other hand, was shown to depend on the nutritional state. The proliferations of retinal vessels, characteristic of this phase, were depressed by continued starvation during the stay in air, whereas the proliferation of hyaloid vessels was not influenced. The normal appearance of the retinal layers of these animals was also remarkable. The retinal vascular proliferations were most common in animals starved during the stay in oxygen but allowed to regain weight during the subsequent stay in air.

2. Other Investigators

Michaelson *et al.* (68) also noted an inhibited vascular outgrowth in under-developed oxygen-exposed animals.

3. Clinical Experience and Applicability of Animal Experiments

The rate of weight gain is no factor of primary importance as to the incidence of RLF (Hardy, 36; Unsworth, 92). Exline and Harrington (27) thought that the dehydration said to accompany the initial neonatal weight loss might be a precipitating factor. This probability is, however, denied by several investigators (*e.g.* LaMotte and Tyner, 60). It is highly questionable as to whether the modifying effect of the state of nutrition found in the experimental disease is in any way applicable to the course of RLF as the deviations from normal weight of the animals were excessive. It is, however, interesting to note the report of Lubchenco *et al.* (64) who found a high incidence of RLF in children with low birth weight, a great initial weight-fall and the subsequent regaining of weight at a relatively fast speed. They would be somewhat analogous to the animal group starved during the stay in oxygen and which regained weight during the stay in air, and these animals showed, as mentioned, the highest incidence of retinal vascular proliferations. No correlation could be demonstrated, on the other hand, between the incidence of RLF and the age at which the birth weight had been recovered (von Winning, 95).

F⁶. Influence of Other Factors on the Experimentally Induced Oxygen Lesions

There have been several attempts to prevent or modify the experimentally induced oxygen lesions. Sympathectomy has not been shown to have any effect in this respect (Patz, 76; Cook and Ashton, 3 b) nor has the addition

of CO₂ to the high oxygen atmosphere (Patz, 76; Ashton *et al.*, 4; Hellström, 38). The administration of the anticoagulant Tromexan modified the degree of vaso-obliteration seen after oxygen exposure and the effect was interpreted as an ability to prevent intravascular coagulation so that the affected vessels were able to reopen in air (Ashton *et al.*, 4). Priscol (Ashton *et al.*, 4) and nicotinic acid (Patz, 76) were without effect.

G. Electrophysiological Studies of the Oxygen Effect

1. *Present Investigation (VII).*

The vasoconstrictive and vaso-obliterative effect of oxygen together with the inhibition of retinal vessel has led to a widely accepted view that this phenomenon causes an ischaemic hypoxia of the retina as mentioned earlier. This electrophysiological part of the present study was undertaken with the view to find out whether this hypothesis might gain experimental support. The question seemed to be of great interest to the understanding of the basic mechanism of oxygen effect. The electroretinogram (ERG) was considered to be a method of investigation reflecting the functional condition of the retina and, from a technical point of view, relatively easy to record.

Kittens were used in this work because their eyes are closely comparable to those of premature infants in regard to the degree of vascular maturation. Other reasons for using kittens were that this animal had been employed in the extensive work of Ashton *et al.* (4) in producing oxygen-induced lesions closely similar to RLF and that it also had been used in the study of the development of the ERG (Zetterström, 97).

In part of the experiments the effect of approximately 12 hours' exposure to 70 per cent oxygen was studied in about 2-week-old kittens. This is an age, where the kittens have developed a recordable ERG and when the sensitivity of retinal vessels to the oxygen effect is maintained. No constant change of the ERG, as judged by the amplitude of the *b*-wave, was registered, following this relatively short duration of oxygen treatment. This is somewhat surprising in view of the potent constrictive effect of oxygen when observed directly with the limbal window method (Ashton and Cook, 2). It points to a preservation of the function of the retina and indicates that the oxygenation of the retina is sufficient for this purpose.

In the main part of the investigation kittens were kept in 70 per cent oxygen from birth up to an age of 3 to 4 weeks and following this exposure the ERG was recorded during continued oxygen administration. A high incidence of abnormal ERG's was found, being of an advancedly negative type, with either a complete absence of the *b*-wave, or an abnormally deep

a-wave, sometimes with an amplitude greater than that of the succeeding *b*-wave. Such an abnormally negative ERG occurred in three-fourths of the eyes examined. It is to be noted that the criteria for the indication of an ERG as abnormal were rigorous and that the proportion of pathological ERG's may have been even higher. Most animals were reexamined during the subsequent days or weeks in air. There was a general tendency towards normalization of the ERG approximately after one week and one to three weeks following the discontinuance of the oxygen exposure almost two-thirds of the recorded eyes had normal ERG's and thus the proportion of normal and abnormal ERG's was reversed. In preliminary investigations with kittens 3-4 weeks old at the onset of the oxygen exposure no similar effect on the ERG could be demonstrated.

The significance of the present study lies in the fact that an injurious effect of oxygen on the retina with functional impairment could be proved during the stay in oxygen, that is during the vaso-obliterative phase of the oxygen effect. There was no evidence indicating an exaggeration of this functional impairment following transfer to air. On the contrary, there was a clear tendency towards normalizing during the subsequent revascularization period or vasoproliferative phase. No attempt was made in the present investigation to correlate the ERG with the ophthalmoscopical fundus picture, but such a study would be of great interest.

It is also known from animal experiments that ligation of the common carotid artery is followed by a negative ERG and it would seem logical to interpret this finding following oxygen exposure and vaso-oblation in kittens as a sign of deprivation of the oxygen supply; an ischaemic hypoxia. This conclusion is, however, not definitely justifiable as metabolic poisons may cause a similar changes of the ERG (Noell, 69; Praglin *et al.*, 79) and these recent experiments by Noell (69) have shown that high oxygen concentrations may cause a visual cell damage in the adult rabbit similar to that caused by such metabolic poisons as iodoacetic acid and X-ray treatment. Irrespective of the primary mechanism the conclusion was drawn from the present electroretinographic study that a retinal damage occurs during the vaso-obliterative phase when the kitten is still in oxygen and that the vasoproliferative phase following transfer to air is electroretinographically the phase of normalization.

2. Clinical Experience and Applicability of Animal Experiments

In clinical electroretinography (Karpe, 53; Karpe and Uchermann, 54; Henkes, 39, 40) negative ERG's have been reported e.g. in acute circulatory disturbance of the retina. No study has been reported, where premature children with acute stages of RLF or oxygen-exposed infants have been

examined with the aid of ERG. An extinguished ERG has been demonstrated in cicatricial stages (Zetterström, 98). As stated previously there are, however, many convincing similarities between the oxygen-induced lesions in the kitten and early human RLF and these strengthen the applicability of the present electroretinographic findings to the patho-physiologic effect of oxygen on the premature infant's retina. This would imply that when the vasoproliferative stage starts in the premature eyes, the injurious oxygen effect is already established.

H. Metabolic Studies of the Retina in Oxygen-Exposed Animals

The basic mechanism of oxygen action on the immature retina has been much discussed, although most authorities favour the view that the injurious effect is secondary to the vascular changes. In that case the almost selective sensitivity of retinal vessels remains to be explained and the question is intimately connected with the unknown mechanism regulating the growth of retinal vessels. An opposite view explains the action of oxygen as a primarily metabolic poisoning. This latter view and speculations on this problem had so far not been experimentally tested.

1. *Histochemical Enzyme Study (IX).*

A poisoning of sulfhydryl-containing enzymes has been suggested as one of the possible pathways for the postulated metabolic injury. This theory is mainly based on the *in-vitro* studies made by Dickens (25). One of the enzymes inhibited through high oxygen tension in his work was succinic dehydrogenase, which may be demonstrated by means of a histochemical method introduced by Seligman and Rutenburg (84) and modified by Padykula (72). In the present paper the effect of oxygen exposure on the histochemical localization of this enzyme in the immature retina was investigated. In the adult retina of different species enzyme activity was concentrated to the inner segments of the bacillary layer and was less intense in the ganglion cell and plexiform layers. The development of activity in the immature retina was also studied in ratlings. At birth there was almost no sign of activity but during the first weeks of life the activity was found to increase successively and the adult pattern was established at nine days of age.

The influence of oxygen on this development was studied in ratlings and young mice, continuously exposed from birth up to the age of one month. These oxygen-exposed animals showed no signs of abnormal distribution or altered intensity of succinic dehydrogenase activity.

In making this statement the limitations of a histochemical method as

to quantitative differentiations must be kept in mind. The question, as to whether oxygen exerts a toxic effect on the retina through inhibition of enzymes such as succinic dehydrogenase, can thus not be definitely settled on the basis of the present histochemical work. There is, on the other hand, no positive evidence, as judged by the localization of enzyme activity, that such a mechanism is present.

2. *In Vitro Metabolic Studies (X).*

The special metabolic qualities of the retina are reflected in a very high *in vitro* oxygen consumption and a high, especially anaerobic, glycolysis. In this respect the retina is distinguished from any other tissue or organ. This remarkable feature has been connected with the selective effect of oxygen on the retina. Brehme (14, 15) has suggested that an increased oxygen supply interferes with the anaerobic glycolysis of the retina and this glycolysis is postulated to be essential also *in vivo*, especially for the immature retina. The constriction of retinal vessels is interpreted as a compensatory mechanism considered to counteract this oxygen effect.

In this part of the investigation newborn ratlings were continuously kept in 70 per cent oxygen from birth up to an age of 5–7 weeks. The metabolism of the retina was studied with manometric methods, in regard to the oxygen consumption and anaerobic glycolysis. The observed differences between oxygen-exposed and control animals were small and insignificant. Consequently the hypothesis signifying oxygen as the cause of primary, metabolic disturbance of the retina did not gain support.

The manometric methods also have their significant limitations and the *in vitro* metabolism is not always a reliable index as to the conditions *in vivo*. This fact reduces the value of the above conclusion to some extent.

I. The Influence of Oxygen on Ocular Structures Other than the Retina

In the morphological part of the present investigation the effect of oxygen on the hyaloid vessels has also been noted. A conspicuous inverse relationship between the hyaloid and retinal vessels was pointed out and this observation has been confirmed by others (Patz, 75; Gerschman *et al.*, 32). Proliferating hyaloid vessels in connection with inhibited retinal vascularization was frequently seen in animals continuously exposed. They were especially impressive in the tunica vasculosa lentis and were also often seen to creep along the vitreal surface of the retina, sometimes apparently sending branches through the internal limiting membrane into the superficial layers of the same. The impression was that the hyaloid vessels were pro-

liferating as a compensatory mechanism, contributing to the vascular supply of the retina. Especially on transfer to air they were dilated and frequently the source of vitreous haemorrhages. During the stay in air the hyaloid vessels successively regressed.

As for the other vascular system of the eye the choroid vessels did not reveal any constant changes but were, on the other hand, not easy to study in routine histologic sections due to technical difficulties and the pigmentation of the choroid. The vessels of the iris were often remarkably dilated and sometimes the possible source of haemorrhages entering the anterior chamber.

The changes of the hyaloid vessels are of less importance regarding the applicability to RLF, as the bulk of hyaloid vessels have regressed in the premature infant. Engorged vessels of the iris, on the other hand, have been described by *e.g.* von Winning (95) and Huggert (44). Increased vascularization of the iris has also been demonstrated in pathological specimens of RLF (Reese and Blodi, 81).

The effect of increased oxygen concentration on the growth of newly formed vessels in the cornea of adult rabbits has been studied by Michaelson *et al.* (67). By means of a thermal lesion the cornea was injured and a pathologic corneal vascularization induced. Increasing the concentration of oxygen in the air breathed to 50 per cent did not affect the extent of new vessel growth. Similar experiments were carried out by Ashton and Cook (2), who induced corneal vascularization in the rabbit by intracameral injection of alloxan. Oxygen in concentrations at 70–80 per cent had no influence on the extent and density of new vessel formation.

J. The Influence of Oxygen on Structures Other than the Eye

It is beyond the scope of the present discussion to give a detailed account of the attempts to demonstrate oxygen-induced lesions in organs other than the eye and this work will only be briefly mentioned. That pulmonary changes occur following the inhalation of high concentrations of oxygen is well known (Bean, 5; Paine *et al.*, 73; I) and evidence has been brought forward that this is due to capillary damage (Berfenstam and Zettergren, 8). Capillary growth induced in the rabbit's ear was not significantly altered by oxygen administration (Ashton and Cook, 2). Patz (76) states that "extensive examination of all other organs in mice, rats and kittens have failed to reveal changes produced by oxygen in other organs, except for occasional pulmonary oedema on prolonged exposure". Inhalation of oxygen under pressures greater than one atmosphere causes mainly cerebral symptoms (Donald, 26).

K. General Summary of the Oxygen Effect on the Immature Animal Eye and the Application to the Pathogenesis of Retrolental Fibroplasia

The experiences gained from the present investigation and those made mainly by Ashton *et al.* (2, 3 a, 3 b, 4) and Patz *et al.* (75, 76, 78) allow the following conclusions to be drawn as to the mechanism of oxygen effect. Oxygen causes an inhibition of the retinal vascularization, and constriction and obliteration of already developed retinal vessels. The effect may be induced with concentrations as low as 40 per cent, with higher concentrations the incidence rapidly increases. The critical duration is probably a few days, possibly even shorter. Revascularization is frequently excessive and abnormal with proliferating vessels of the nerve-fibre layer budding into the vitreous body. This vasoproliferative phase occurs chiefly on transfer to air, but minor proliferations may occur in oxygen, especially when lower concentrations are used. The vascular changes occur only in eyes with incomplete vascularization. The proliferative changes may be delayed through a process of slow weaning, but the other pathologic changes of the eye are not influenced. Among factors influencing both normal and pathologic vascularization is the state of nutrition.

Prolonged continuous oxygen exposure may lead to atrophic changes of the retina, but mainly of the inner layers. Further evidence indicating a lesion during the stay in oxygen comes from electrophysiological investigations, with the animals frequently showing an abnormal ERG, normalizing during the vasoproliferative phase on transfer to air.

There is no experimental evidence that oxygen causes an enzyme inhibition postulated to lead to histotoxic anoxia. Nor is the theory of a primary, metabolic disturbance of the retinal cells supported. General hypoxia seems to play no rôle.

It is possible to explain the structural changes of the retina as secondary to the vascular with atrophy following prolonged obliteration and inhibition and with thickening and irregularities during the phase of vasoproliferation. This is also supported by the time-table of the changes: signs of vascular inhibition are always present before atrophy of retinal layers can be demonstrated. It must be pointed out, however, that the routine histologic methods used in the present investigation do not allow any conclusions as to more subtle degenerative changes of retinal cells. A complementary study using more refined methods is necessary for further detailed and complete evaluation of retinal structures.

Applied to the pathogenesis of RLF these experimental findings give a clue to the mechanism of the oxygen effect. The primary lesion occurs

during the oxygen exposure together with, and probably due to, vascular changes causing ischaemia. The probable critical levels and duration of oxygen have already been mentioned. The margin between the oxygen content of air and the 30–35 per cent level could be used for slow weaning tests. The vascular proliferations may be considered as a reactive stage. The primary lesion might be caused by factors other than oxygen exposure, although the experimental evidence for this is meagre. This is in accordance with the observation of cases, where no supplemental oxygen has been given (*e.g.* Owens, 70; Coxon, 21; Dancis *et al.*, 24; Bjelkhagen, 10; Castrén, 19).

The animal experiments offer no satisfactory explanation of the progressive course of the reactive, vasoproliferative stages of the disease in premature infants, and further experimental work, with a view to exploring the conditions leading to vitreous organization and the mechanism of retinal detachment, is urgently needed.

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Addendum: A recent review is given in Report of the sixteenth M & R Pediatric Research Conference: Retrolental Fibroplasia: Role of Oxygen, 1955.

Experimental Approach to the Pathogenesis of Retrolental Fibroplasia

X. In Vitro Metabolism of the Retina of Oxygen-Exposed Ratlings¹

by

BO E. HELLSTRÖM

In previous papers the effect of oxygen exposure of young animals on the vascularization and development of the retinae has been reported (Gyllenstein and Hellström, 8, 9, 10; Hellström, 11, 12, 14). From these and other morphological studies (Ashton *et al.*, 1; Patz *et al.*, 22, 23) it seems clear that continuous oxygen exposure causes inhibition of the outgrowth of retinal vessels together with constriction and obliteration of those already developed. Changes produced in the electroretinograms of oxygen-exposed kittens were also well compatible with circulatory disturbances of the retina (Hellström, 13). In mice, the final stages of the oxygen-induced disease of the retina, consist of atrophy mainly of the inner layers of variable intensities (Hellström, 14).

It seems probable that the inhibited vascularization induced in these experiments is of a primary nature and that the atrophy follows secondarily. It has, however, been suggested that the mechanism of the oxygen effect should lie in a primary, metabolic injury of retinal cells (Ingalls and Purscott, 17; Patz, 22), brought about by interference with sensitive oxidative enzymes leading to histotoxic anoxia. In a recent work, however, oxygen exposure did not influence the histochemical distribution of succinic dehydrogenase of the retina (Hellström, 15).

Another way of gaining information concerning a disturbed metabolism in biological material is through manometric methods, introduced by Warburg, and the retina is especially suitable for this purpose. The conspicuous metabolic characteristics of the retina are reflected in a great oxygen consumption and also in a high rate of glycolysis, particularly the anaerobic. Based on this knowledge Brehme (3, 4) has brought forward a hypothesis that an increased

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oxygen supply tends to decrease the anaerobic glycolysis, said to be essential for the retina, and this effect is counteracted by the constriction of retinal vessels.

The purpose of the present investigation was to study the *in vitro*-metabolism of the retina in oxygen-exposed ratlings. Retinal vascular changes have been produced in this animal by continuous oxygen exposure at a relatively high percentage (Patz *et al.*, 23). The information gained by such a study may either support or contradict the theory of a primary, metabolic injury and possibly aid in solving the problem of oxygen effect on the immature retina.

Material and Methods

Newborn ratlings with their mothers were raised in special chambers described previously (Gyllensten and Hellström, 8). The flow of the oxygen-nitrogen mixture was 0.5 litres per minute, the concentration of oxygen was kept at 70 per cent as measured with a Beckman oxygen analyzer. The range of fluctuations of the oxygen concentrations was ± 2 per cent. Soda lime was used to absorb CO_2 , the concentration of which was not measured. The humidity was close to 100 per cent, the temperature ranged between 20 and 25°C. The animals were fed and handled as described elsewhere (8). No interchange of the mothers was necessary. The exposure was continued for 5–7 weeks. Control animals were raised in ordinary cages. The weight and the general condition of the oxygen-exposed ratlings did not seem to differ from those of control animals of the same age. In two experiments 11 ratlings were suffocated due to accidental discontinuance of the oxygen supply, otherwise there were no deaths either among the experimental or among the control animals.

124 retinæ from 62 oxygen-exposed animals were prepared, 60 of them being used to measure the oxygen consumption, 44 for anaerobic glycolysis and 20 to determine the dry-wet weight ratio. 126 retinæ from 63 control animals were also examined, 50 of them for oxygen consumption, 50 for anaerobic glycolysis and 26 for the dry-wet weight ratio.

The animals were killed by decapitation and the eyes were enucleated. The preparation took place with the aid of a binocular dissecting microscope with the eyes placed in a cooled substrate-fluid of the same composition as was used in the manometric estimation to follow. The dissection took place in full day-light. The bulbs were opened coronally near the ora serrata, the retinæ isolated by blunt dissection, and remnants of the vitreous body were carefully removed. Following the determination of the wet weight two retinæ were put into each Warburg-flask. The whole procedure from

the killing of the animals until the moment when both retinae were put into the flasks took about 10 minutes.

It was found necessary to calculate the dry weight of the retinae used in the experiments, on the basis of dry-wet weight ratio, as the retina was partly reduced to tiny fragments during the shaking and was found to lose about one third of its initial wet weight during a few hours of the same.

Manometric methods. — Oxygen consumption: It has been pointed out by Røe (25) that the Krebs-Ringer phosphate solution commonly used in respiration experiments (Umbreit *et al.*, 27) has an insufficient buffer capacity when the retina is studied, and he recommended carbonate-free horse serum as a substrate solution. When used undiluted in the present experiments this medium easily formed bubbles, which were contaminated with alkali from the filtering paper in the central cup. Because of this the carbonate-free horse serum adjusted to a pH of 7.0 was diluted with equal amounts of a Krebs-Ringer phosphate solution containing 0.2 per cent of glucose. 3 c.cm of this mixture was found to have a sufficient buffer capacity. 0.2 c.cm of a 20 per cent potassium hydroxide solution was used in the centre cup. pH of the substrate was measured before and after each experiment. The temperature of the water-bath ranged between 36.9 and 37.1°C, in each experiment the range of fluctuation was 0.10°C or less. Air was used as the gas phase. The shaking rate was 100 cycles per minute. Following 15 minutes of equilibration the oxygen consumption was measured at hourly intervals. The average of the consumption during the first two hours was accepted as the significant value and the Q_{O_2} , that is the oxygen consumption during one hour in c.mm per mg dry weight was calculated.

Anaerobic glycolysis. — As substrate the Krebs-Ringer carbonate solution (Umbreit *et al.*, 27) with the addition of 0.2 per cent of glucose was used. A mixture of 95.3 per cent of nitrogen and 4.7 per cent carbon dioxide comprised the gas phase. To attain a sufficient purity of the nitrogen, which originally had a content of 0.01–0.05 per cent of oxygen, the mixture was led through an apparatus, where active copper adsorbed to diatomaceous earth was heated to 200°C (Meyer and Ronge, 20). This procedure did not affect the content of CO₂, as analyzed in the gas laboratory of AGA. The temperature ranges of the water-bath and the shaking rate were the same as in the respiration measurements. Following equilibration, the CO₂ produced during the first hour was measured and used to calculate the $Q_{CO_2}^{N_2}$, that is the amount of CO₂ in c.mm driven out from the substrate solution in one hour per mg of dry weight during anaerobic conditions.

Results

The dry-wet weight ratio was found to be 0.14 in both oxygen-exposed and control animals. The results of both oxygen consumption and anaerobic glycolysis have been tabulated (Table 1). In this Table the mean values of each group have been calculated together with the standard error of the means. It can be seen that the observed differences between oxygen-exposed and control animals are small and insignificant both concerning oxygen consumption and anaerobic glycolysis.

TABLE 1
Metabolic *in-vitro* studies with isolated retinæ.

Oxygen consumption (Q_{O_2})	Number of animals	Mean Q_{O_2}	Standard error of the mean
Oxygen-exposed animals	30	18.6	± 1.6
Control animals	25	15.7	± 1.7
Difference with standard error		2.9 ± 2.3	
Anaerobic glycolysis ($Q_{CO_2}^{N_2}$)	Number of animals	Mean $Q_{CO_2}^{N_2}$	Standard error of the mean
Oxygen-exposed animals	22	43.9	± 2.1
Control animals	25	47.5	± 2.0
Difference with standard error		-3.6 ± 2.9	

Discussion

No systematic investigation has been published which has dealt with the respiration of the developing retina postnatally. To judge from experiences with the brain of the rat and other species (Weill and Mandel, 29; Tyler and Van Harrevald, 26; Himwich and Fazekas, 16), there seems to be reason to believe that adult levels have been reached or almost reached at an age of 5-7 weeks. As for the oxygen consumption the values gained from the present investigation are in good agreement with those found by several other investigators (e.g. Dickens and Greville, 7; Laser 18, 19; Craig and Beecher, 5; Robbie and Leinfelder, 24) who used adult rats. The figures for anaerobic glycolysis are lower than those presented by e.g. Warburg *et al.* (28) and Dickens and Greville (7). There may be several reasons for this discordance, but as the chief aim of the present investigation was to compare the

values of two groups, where an identical technique has been used, it seems of less importance.

The results of the present investigation do not support the speculations as to a primarily disturbed cellular metabolism of the retina through the effect of oxygen exposure of the animal. When this conclusion is drawn, however, some limitations of the method used have to be kept in mind. As demonstrated e.g. by Dawson (6) oxygen uptake may not always be a good index of a normal metabolic activity of the cell. Injection of the metabolic poison iodoacetate in rabbits caused visual cell damage with simultaneous decrease of glycolysis (Berardini, 2; Noell, 21), whereas high intensity X-radiation did not change the glycolytic activity in spite of the fact that a similar morphological injury was produced (Noell, 21).

The effect of exposure to high concentrations of oxygen was also explored in Noell's (21) investigation and it led to similar visual cell damage. This stands in contrast to the picture of atrophy, preferably of the inner layers of the immature retina in connection with vascular changes, found in my own experiments with mice (Hellström, 14). The findings by Noell show that, at least in the rabbit, also the *mature* retina may be injured by oxygen, when used in high concentrations, and this injury may be "primarily metabolic". No metabolic studies to confirm this were, however, reported.

It is also known that the sensitivity of the immature retina to oxygen varies among different species. It was observed during the isolation of the retinæ in the present experiments, that the inhibition of the vascularization was far from complete. The same experience was gained by Ashton *et al.* (1) who found a less regular and extensive vaso-obliteration in ratlings when compared with kittens as judged by injection technique.

It would be of great interest to extend the metabolic experiments to kittens and try to correlate the fundus picture, electrophysiological and metabolic findings with the morphological picture. Such a study would probably add considerably to our present knowledge concerning the oxygen effect on the immature retina, a knowledge that might be of great value to the understanding of the pathogenesis of retrolental fibroplasia in premature infants.

Summary

1. Ratlings 5-7 weeks of age were kept in 70 per cent oxygen from birth.
2. The retinæ were studied in vitro with determinations of oxygen consumption and anaerobic glycolysis.
3. There was no change indicating a metabolic injury of respiration or glycolytic activity, as compared with control animals.
4. The significance of this finding regarding the mechanism of oxygen injury to the immature retina is discussed.

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